# Calprotectin play a Role as a DAMP for Development of Tubulointerstitial Injury Accompanied with MPO-ANCA-Associated Glomerulonephritis

Ai UCHIDA, Naoko HIMURO, Maho WATANABE, Katsuhisa MIYAKE, Kenji ITO, Tetsuhiko YASUNO, Yasuhiro ABE, Aki HAMAUCHI, Kousuke MASUTANI, Hitoshi NAKASHIMA

Division of Nephrology and Rheumatology, Department of Internal Medicine, Faculty of Medicine, Fukuoka University

**Abstract**

**Background.** Glomerular endocapillary inflammation may proceed to crescent formation and exuded protein leakage, which may induce tubulointerstitial injury (TII) in glomerulonephritis. Therefore we naturally think the grade of crescentic formation should relate with the severity of TII. However, it is well known that the severity of TII was not correlated with that of glomerulonephritis in anti-neutrophil cytoplasmic antibody (ANCA)-associated crescentic glomerulonephritis (ANCAGN). Activated neutrophils by ANCA have been shown to release neutrophil extracellular traps (NETs), and which plays a role for a trigger of sterile inflammation using the Nod-like receptor family pyrin domain–containing-3 (NLRP3) inflammasome. The aim of this study was to indicate the sterile inflammation is a distinct mechanism of TII in ANCAGN.

**Methods.** Twenty-two myeloperoxidase-ANCA-positive ANCAGN patients were enrolled, and their kidney biopsy specimens were evaluated with regard to glomerular injury (GI) and TII. For a disease control group 25 patients with crescentic IgA nephropathy were also enrolled. GI was represented by cellular crescent formation rate (cCFR), and TII was exhibited by cellular TII (cTII), which is the average number of infiltrating cells. The expressions of calprotectin in the biopsy specimens were immunohistologically evaluated. The mRNA expressions of inflammasome related molecules were examined.

**Results.** The correlation between the values of cCFR and cTII was recognized in IgAN, but

not in ANCAGN. However, the positivity of calprotectin was correlated with the value of cTII in ANCAGN. The mRNA expression of Interleukin-1β was significantly correlated with the value of cTII in ANCAGN. The Toll-like receptor 4 (TLR4) and NLRP3 also correlated in ANCAGN. Immunohistochemical analysis demonstrated that calprotectin, TLR4 and NRLP3 proteins were positively stained in the severe infiltrating area in ANCAGN.

**Conclusions.** Calprotectin produced by ANCA activated neutrophils may behave as damage-associated molecular patterns in the sterile inflammation in ANCAGN. These results indicated that NLRP3 inflammasome may play a characteristic role in the development and deterioration of TII in ANCAGN.

**Keywords**: anti-neutrophil cytoplasmic antibody (ANCA), tubulointerstitial injury (TII), interleukin-1β (IL-1β), damage-associated molecular patterns (DAMP), nod-like receptor family pyrin domain–containing-3 (NLRP3) inflammasome

**Footnotes**

Corresponding author: Katsuhisa Miyake

Division of Nephrology and Rheumatology, Department of Internal Medicine, Faculty of Medicine, Fukuoka University

7Chome-45-1 Nanakuma , Jyounan ,Fukuoka

Tel : 092-801-1011 ( 3374 )

Fax : 092-873-8008

Email : md160002@cis.fukuoka-u.ac.jp

## Introduction

In elderly people, myeloperoxidase (MPO) and proteinase-3 (PR3) anti-neutrophil cytoplasmic antibody (ANCA)-associated crescentic glomerulonephritis (ANCAGN) is a leading cause of rapidly progressive glomerulonephritis (RPGN), and the principal histopathological features are glomerular extracapillary proliferation (crescents) and fibrinoid necrosis[1),2)]. Glomerular damage is accompanied by tubulointerstitial damage and generally accepted that the former causes the latter. We naturally think the grade of crescent formation should relate with the severity of tubulointerstitial injury (TII), however, several cases reported in which only TII is noted without any apparent glomerular lesions[3),4)], and it is also well known that the severity of TII was not correlated with that of glomerulonephritis in ANCAGN[5),6)].

IgA nephropathy (IgAN) is another kidney disease exhibits representative cresentic glomerulonephritis. IgAN is the most common primary glomerulonephritis worldwide, and crescent formation is a common histopathological finding, occurring in approximately 20–50% of the patients[7)-10)]. It was clearly showed that crescent lesions are independently associated with a poor renal outcome in untreated patients and even those treated with immunosuppressive therapy[11)]. Most nephrologists realized renal progression correlates more closely with the severity of tubulointerstitial lesions than with the grade of glomerular lesions[12)].

The most popular notion for the mechanism of TII associated with glomerulonephritis is that protein leakage in injured glomeruli into Bowman's space leads to excessive protein reabsorption in proximal tubules[13] . Adding to this direct damage the exudates and dying cellular fragmentation into tubular urine resulted from crescent formation may also act as DAMPs and activate Nod-like receptor family pyrin domain–containing-3 (NLRP3) inflammasome in dendritic cells (DCs) and macrophages harboring in the tubulointerstitium. As a result TII is developed and progressed. Especially ANCAGN has distinctive pathological mechanism compared with other glomerulonephritis such as IgAN, and characteristic activated neutrophils should attack not only glomerular capillaries, but also interstitial blood vessels. These injuries may activates NLRP3 inflamasome to attack tubulointerstitium from the start[14] .

Calprotectin is a heterodimer complex of two intracellular calcium-binding proteins S100A8 and S100A9, and expressed by neutrophils, monocytes and early differentiated macrophages[15), 16)]. It is demonstrated that phagocytes released this complex after their interaction with activated, inflamed endothelium[17)]. Calprotectin is a Toll-like receptor 4 (TLR-4) ligand, and classed as a DAMP and initiates and perpetuates the immune response. In the current study we showed that the expression of calprotectin has more close relation to TII in ANCAGN rather than that in IgAN. The aim of this study is to clarify that calprotectin

released by ANCA activated neutrophils plays a role as a DAMP for activating NLRP3 inflammasome, and which develops and deteriorate TII.

This study was performed in accordance with the Declaration of Helsinki and was approved by the ethics committee of Fukuoka University. Written informed consent was obtained from all patients.

## Materials and Methods

### 1, Patients

We enrolled 22 patients who were diagnosed with MPO-ANCA-positive ANCAGN, histologically diagnosed crescentic glomerulonephritis and 25 patients with crescentic IgAN in the Division of Nephrology and Rheumatology in Fukuoka University Hospital between January 2015 and December 2018. All ANCAGN patients received kidney biopsy examinations within 31.2 ± 17.8 days since subjective symptom appeared. While some IgA patients received biopsy on standby. No steroid or immunosuppressant was prescribed at the time of kidney biopsy for all enrolled patients. The clinical background data of the patients are shown in Table 1.

### 2, Histological evaluations

The diagnosis was determined according to the classification of the Japanese Renal Biopsy Registry based on the classification of glomerular diseases [18]. Biopsy specimens were processed and observed using routine methods, including light microscopy, immunofluorescence techniques, and electron microscopy. ANCAGN was defined according to laboratory and histological findings of rapidly progressive glomerulonephritis along with positive tests for MPO-ANCA.

The severities of cellular crescents formation of glomerulus and cTII were evaluated in a blind manner by histologic examination with periodic acid-Schiff staining and periodic acid-methenamine-silver staining. A crescent occupied more than half of the total area by cellular elements was defined as a cellular crescent, and the percentages of glomeruli showing cellular crescent for all glomeruli were expressed as an index of the cellular crescent formation rate (cCFR). Concerning cellular TII (cTII) were evaluated as follows; cTII was an index defined as the average number of infiltrating cells for five high power field (HPF) areas in order from having the most infiltrating cells in tubulointerstitium. The observation areas were selected under eliminating the overlap.

**3, Reverse transcription-polymerase chain reaction (PCR)**

Total RNA was extracted from the kidney biopsy specimens collected before commencing steroid therapy, using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA).

Quantitative cDNA amplification was performed according to the manufacturer's instructions. All samples were stored at –20 °C until use. cDNAs of the cytokines were analyzed by real-time PCR using Power SYBR Green PCR Master Mix (Applied Biosystems Japan, Tokyo, Japan) or TaqMan Gene Expression Master Mix (Applied Biosystems Japan, Tokyo, Japan). Sequence-specific amplification was detected with an increased fluorescence signal during the amplification cycles, using an ABI Prism 7500 sequence detection system (Perkin Elmer Japan, Yokohama, Japan). To provide a meaningful comparison between different samples, we calculated the amount of PCR products relative to the amount of β-actin in each sample. Oligonucleotide primers and probes were designed using the Primer Express program (Applied Biosystems Japan) or purchased directly. Primers sequences for RT-PCR using SYBR green chemistry are follows. β-actin; 5′ GCA AAG ACC TGT ACG CCA AC 3′ and 5′ CTA GAA GCA TTT GCG GTG GA 3′, NLRP3; 5' GAA GAA AGA TTA CCG TAA GAA GTA CAG AAA 3' and 5' CGT TTG TTG AGG CTC ACA CTC T 3'. Purchased Taqman primers were as follows: Hs01060665-g1 for TLR4, Hs01555410-m1 for interleukin-1β (IL-1β) .

**4, Immunohistological analysis**

After deparaffinization in xylene and ethanol, and washing in phosphate-buffered saline (PBS), paraffin-embedded sections were incubated with mouse anti-human TLR-4 antibody (Ab), rabbit anti-human nucleotide-binding domain, leucine-rich–containing family,

pyrin domain–containing-3 (NLRP3) Ab, and mouse anti-human calprotectin MRP8/14 (Bachem, Bubendorf, Switzerland) at a concentration of 1 μg IgG /ml PBS, including 1% bovine serum albumin or 5% normal goat serum. Monoclonal and polyclonal staining was performed using anti-mouse or anti-rabbit IgG horseradish peroxidase-labeled polymer (Dako Cytomation, Inc., Carpinteria, CA, USA). A part of immunofluorescent study was performed with OPAL-7-plex reagents, imaged at 20X on the Mantra Quantitative Pathology Imaging System, and analyzed using inForm software (all from Perkin-Elmer, Waltham, MA, USA).

Calprotectin positivity was evaluated as follows. The average number of positive cells of five glomeruli in order from having the most abundant positive cells was defined as glomerular calprotectin positive score (GCP score). Similarly, tubulointerstitium calprotectin positive score (TICP score) was defined for five HPF areas in tubulointerstitium. The observation areas were selected under eliminating the overlap.

**5, Statistical analysis**

Statistical significance of the differences between groups was determined by Pearson's rank correlation coefficient. All statistical analyses in this study were performed using SPSS statistics software version 23. *P*-values <0.05 were considered to be statistically significant.

## Results

### 1, Histological evaluation of TII

#### 1) Correlation of the severity between glomerular injury (GI) and TII

We evaluated the relationship between GI and TII. Pearson's correlation coefficient for IgAN was r=0.400 (P-value 0.047), and the value of cTII was correlated with that of cCFR. While correlation coefficient for ANCAGN was r=0.336 (P-value 0.068), and correlation was denied (Fig. 1). This result supported the notion that there is a different mechanism for TII in ANCAGN from that in IgAN.

#### 2) Immunohistological analysis for calprotectin

We evaluated the calprotectin positivity for both ANCAGN (Fig. 2A) and IgAN (Fig. 2B). Within and around a crescent prominent expression calprotectin-positive cells were seen, but not within sclerosed glomeruli in both diseases. Abundant calprotectin-positive cells around a small vessel were apparent in ANCAGN rather than in IgAN (Fig. 2C). GCP scores in ANCAGN and IgAN were 3.34±5.63 and 3.20±3.90, respectively (P-value 0.523). While TICP scores in ANCAGN and IgAN were 9.19±9.10 and 2.4±3.53, respectively(P-value 0.015). The correlation coefficients between GCP and TICP scores in ANCAGN and IgAN were r=0.464 (P-value 0.03) and r=0.541(P-value 0.005), respectively (Fig. 3).

**2, Role of the inflammasome in TII in ANCAGN**

The value of TICP showed a close correlation with cTII (coefficient 0.459, P-value 0.036) (Fig 4A). The expression of *TLR4* was correlated with TICP (coefficient 0.459, P-value 0.036) (Fig 4B). The expressions of *NLRP3* (coefficient 0.365, P-value 0.095) and *IL-1β* (coefficient 0.167, P-value 0.459) were not correlated (data not shown).

While the value of cTII showed the positive correlations with the expression of *TLR4* (coefficient 0.469, P-value 0.028), *NLRP3* (coefficient 0.434, P-value 0.044), and *IL-1β* (coefficient 0.520, P-value 0.013), respectively (Fig. 5).

Immunohistochemical analysis demonstrated that TLR4 protein was stained some tubulointerstitial infiltrating cells. Many NLRP3-positive cells were observed in the severe infiltrating area, and similarly, calprotectin positive cells were scattered in the tubulointerstitium, but the number of these cells was less than one-fifth that of NLRP3-positive cells. Almost all of these positive cells were also positive for CD68 expression (data not shown). Results by Mantra Quantitative Pathology Imaging System showed the distribution of calprotectin, TLR4, and NLRP3-positive cells. Many NLRP3-positive cells and several TLR4-positive cells surrounded calprotectin positive area were shown. However, NLRP3-positive cells were also observed calprotectin negative area such as perivascular region (Fig. 6).

## Discussion

In this study we showed that the correlation between the severity of GI and TII was not recognized in ANCAGN. In general, glomerulonephritis is associated with tubulointerstitial lesions to some extent, and it is universally agreed that glomerular damage is the cause of TII. In fact although the severity of GI and TII were correlated in IgAN, no significant correlation was recognized in patients with ANCAGN (Fig. 1). These results indicated that a distinctive mechanism effected on TII in ANCAGN. A marked characteristic of pathological condition in ANCAGN is that activated neutrophils and monocytes have important role in pathogenesis. ANCAs have been shown to induce neutrophil extracellular traps (NETs)[17),19)] and which are present in kidney biopsies of ANCAGN patients[19), 20)] . Although NETs play an important immune defense role showing an antimicrobial mechanism coexisting with enzyme degranulation and reactive oxygen species production, they express potential autoantigens and have therefore been implicated in the pathogenesis of ANCAGN[21),22)]. Actually NETs consist of expelled chromatin loaded with lysozomal and cytosolic proteases[23)] , and involve massive danger associated molecular patterns (DAMPs). In the extracellular space these DAMPs can bind to pattern recognition receptors (PRRs) or to specialized DAMP receptors to activate the immune system by promoting release of pro-inflammatory mediators[24)]. The immune cells that participate in these processes are mainly DCs and macrophages harboring in the intertubular interstitium. They survey against injury and infection and contribute to organ homeostasis but may also promote the progression of CKD[25)] .

Calprotectin is a heterodimer complex of S100A8 and S100A9, and it has been

widely reported that fecal calprotectin (FC) levels reflect regional inflammation of the gastrointestinal tract, and that the FC level was useful as a reliable surrogate marker of endoscopic and histologic activity in ulcerative colitis[26), 27), 28)]. In the patient with ANCA-associated vasculitis it was demonstrated that calprotectin is up-regulated and represents a marker of cellular immune activation and disease activity[29)]. It was also shown that elevated serum levels of calprotectin may be used as an indicator of severe disease and cardiovascular disease (CVD) in systemic lupus erythematosus (SLE)[30)], and demonstrated platelet calprotectin levels were increased in SLE patients, particularly in those with CVD[31)]. The fact that calprotectin was extracellulary released during NETosis[21), 23)] deserves attention, and this reaction remind us the existence of specific mechanism for TII in ANCAGN. In this study the expressions of calprotectin in the renal biopsies of patients with ANCAGN and IgAN were examined. The conspicuous expression of calprotectin was recognized within active crescents and in areas of focal necrosis, but was absent in sclerotic or normal glomeruli (Fig. 2). TICP and GCP scores were indexes of calprotectin positive cells in TII and glomerulus, respectively, and these were correlated in both diseases. But calprotectin-positive cells in cellular infiltration area in ANCAGN were much abundant compared with that in IgAN (Fig. 3). The values of TICP scores reflected the severities of cTII, and the mRNA expression level of *TLR4*, which is a receptor of calprotectin, was also correlated with the TICP scores (Fig. 4). However, the mRNA expression levels of *NLRP3* and *IL-1β* were not correlated with TICP scores (Fig. 5). On the other hand increase of the values of cTII was incorporate with the gain of message for *TLR4, NLRP3* and *IL-1β* (Fig. 6). The fact that the message of *NLRP3* and *IL-1β* correlated with cTII, but not with TICP may reflect the following context; It is thought that many DAMPs other than calprotectin also may be concerned with this reaction. In addition other mechanisms than sterile inflammation should play some role in TII. The production of IL-1β

is limited to interstitial monocytic phagocytes/DCs that having the components of the NLRP3 inflammasome inside the kidney[32), 33)], and this sterile inflammation with NLRP3 inflammasome mechanism with calprotectin and many other DAMPs may work in TII in ANCAGN. In immunohistologic study some tubulointerstitial infiltrating macrophages showed positive staining for TLR4 and NLRP3. Apparently the areas where calprotectin deposits were surrounded with NLRP3-positive cells or TLR4-positive cells were observed, but the region of accumulation of NLRP3-positive cells without calprotectin deposits also recognized (Fig. 6). This distribution may indicate that not only calprotectin, a varieties of DAMPs contribute to innate immunological response through NLRP3 inflammasome.

From a therapeutic standpoint, developing therapeutic method to regulate the NLRP3 inflammasome is important to treat TII in ANCAGN. Colchicine is a tubulin polymerization inhibitor, and consistently disrupts the assembly of the NLRP3 inflammasome[34)]. Although colchicine is a powerful medication for gout to suppress inflammasome activity, it is toxic for cells and tissues and causes several adverse effects[35)]. Therefore, colchicine is not appropriate for the treatment of chronic inflammatory diseases. Instead it was demonstrated that resveratrol, which is a naturally occurring compound produced by many types[36)], prevents the accumulation of acetylated α-tubulin, suppresses NLRP3-inflammasome assembly, and subsequent inflammatory responses[37)]. It was also reported that MCC950 is a novel, selective small-molecule NLRP3-inflammasome inhibitor with powerful *in vitro* and *in vivo* inhibitory effects, enabling clinically relevant testing in large animal models[38), 39)]. On the other hand, neutralizing IL-1β may also represent a rational strategy for TII in ANCAGN. Anakinra, a recombinant IL-1β receptor antagonist has been successfully used in the treatment of type 2 diabetes, asbestosis, and other conditions in the United States[40)]. It was recently reported that Canakinumab is a human anti- IL-1β monoclonal antibody and

reduced major adverse cardiovascular event rates among high-risk atherosclerosis patients with CKD[41]. The prognosis of ANCAGN mainly depends on improvement of GI and TII, and thus targeting inflammasome or IL-1β appears to be a promising strategy to ameliorate TII and prevent interstitial fibrosis.

The present study had several limitations. Firstly, the study was performed at a single center, and histological severity grades and score were evaluated using self-defined definition. Secondary we had to match some condition to compare between ANCAGN and IgAN, which are completely different diseases in patient population and clinical stage, thus this study involved a limited number of patients, particularly those with crescentic IgAN. Finally we could not present inflammasome activity anymore than the expression of mRNA of inflamasome-related molecules.

In summary, calprotectin is a useful surrogate marker for ulcerative colitis, and its role for DAMPs is watched with interest. In ANCAGN calprotectin produced activated neutrophils may behave as a DAMPs to activate NLRP3 inflammasome in the DCs and macrophages within the tubulointerstitium. These reactions induce the release IL-1β, and which may result in development and deterioration of TII. NLRP3-inflammasome inhibitor or neutralizing IL-1β may be excellent strategies to suspend the progress of TII and improve the prognosis of CKD resulting from ANCAGN.

**Acknowledgments**

**References**

1 ) Jennete JC TD : Pauci-immune and antineutrophil cytoplasmic autoantibody-mediated crescentic glomwrulonephritis and vasculitis. Lippincott Williams & Wilkins ( Philadelphia ) , 2007

2 ) Seshan SV , D'Agati VD , Apple GA , Chung J : Renal vasculitis: cllasiffication and atlas of tubulointerstitial and vascular diseases. Williams & Wilkins ( Baltimore ) , 1999

3 ) Nakabayashi K, Sumiishi A, Sano K, Fujioka Y, Yamada A, Fujioka Y, Yamada A, Karube M, Koji H, Arimura Y: Tubulointerstitial nephritis without glomerular lesions in three patients with myeloperoxidase-ANCA-associated vasculitis. Clin Exp Nephro l13: 605-613, 2009.

4 ) Yoshida M, Sasaki M, Nakabayashi I, Akashi M, Tomiyasu T, Yoshikawa N, Kojima T, Ohno N, Yamada M : Two types of myeloperoxidase-antineutrophil cytoplasmic autoantibodies with a high affinity and a low affinity in small vessel vasculitis. Clin Exp Rheumatol27: S28-32, 2009.

5 ) Jennette JC, Falk RJ, Hu P, Xiao H:Pathogenesis of antineutrophil cytoplasmic autoantibody-associated small-vessel vasculitis. Annu Rev Pathol8: 139-160, 2013.

6 ) Hauer HA, Bajema IM, van Houwelingen HC, Ferrario F, Noël LH, LH,Waldherr R, Jayne DR, Rasmussen N, Bruijn JA, Hagen EC: Renal histology in ANCA-associated vasculitis: differences between diagnostic and serologic subgroups. Kidney Int61: 80-89, 2002.

7 ) Edstrom Halling S, Soderberg MP, Berg UB: Predictors of outcome in paediatric IgA nephropathy with regard to clinical and histopathological variables (Oxford

classification). Nephrol Dial Transplant27: 715-722, 2012.

8 ) Haas M.: Histologic subclassification of IgA nephropathy: a clinicopathologic study of 244 cases. Am J Kidney Dis29: 829-842,1997.

9 ) Katafuchi R, Ninomiya T, Nagata M, Mitsuki K, Hirakata H: Validation study of oxford classification of IgA nephropathy: the significance of extracapillary proliferation. Clin J Am Soc Nephrol6: 2806-2813,2011.

10 ) Walsh M, Sar A, Lee D, Yilmaz S, Benediktsson H,Manns B, Hemmelgarm B: Histopathologic features aid in predicting risk for progression of IgA nephropathy. Clin J Am Soc Nephrol5: 425-430, 2010.

11 ) Haas M, Verhave JC, Liu ZH, Alpers CE, Barratt J, Becker JU, Cattran D, Cook HT, Coppo R, Feehally J, Pani A, Perkowska-Ptasinska A, Roberts IS, Soares MF, Trimarchi H, Wang S, Yuzawa Y, Zhang H, Troyanov S, Katafuchi R:A Multicenter Study of the Predictive Value of Crescents in IgA Nephropathy. J Am Soc Nephrol28: 691-701,2017.

12 ) Rychlik I, Andrassy K, Waldherr R, Zuna I, Tesar V, Jancová E, Stejskalová A, Ritz E: Clinical features and natural history of IgA nephropathy. Ann Med Interne (Paris)150: 117-126,1999.

13 ) Kriz W, Hähnel B, Hosser H, Ostendorf T, Gaertner S, Kränzlin B, Gretz N, Shimizu F, Floege J: Pathways to recovery and loss of nephrons in anti-Thy-1 nephritis. J Am

Soc Nephrol14: 1904-1926,2003.

14 ) Edgeworth J, Freemont P, Hogg N : Ionomycin-regulated phosphorylation of the myeloid calcium-binding protein p14. Nature342: 189-192,1989.

15 ) Nacken W, Roth J, Sorg C, Kerkhoff C : S100A9/S100A8: Myeloid representatives of the S100 protein family as prominent players in innate immunity. Microsc Res Tech60: 569-580,2003.

16 ) Frosch M, Strey A, Vogl T, Wulffraat NM, Kuis W, Sunderkötter C, Harms E, Sorg C, Roth J:Myeloid-related proteins 8 and 14 are specifically secreted during interaction of phagocytes and activated endothelium and are useful markers for monitoring disease activity in pauciarticular-onset juvenile rheumatoid arthritis. Arthritis Rheum43: 628-637,2000.

17 ) Kessenbrock K, Krumbholz M, Schönermarck U, Back W, Gross WL, Werb Z, Gröne HJ, Brinkmann V, Jenne DE: Netting neutrophils in autoimmune small-vessel vasculitis. Nat Med15: 623-625,2009.

18 ) Sugiyama H, Yokoyyama H, Sato H, Saito T, Khoda Y, Nshi S, Tsuruya K, Kiyomoto H, Hattori M, Oka K, Kagami S, Nagata M, Kawamura T, Honda M, Fukasawa Y, FUkatsu A, Morozumi K, Yoshikawa N, Yuzawa Y, Matsuo S, Kiyohara Y, Joh K, Taguchi T, Makino H: Japan Renal Biopsy Registry: the first nationwide, web-based, and prospective registry system of renal biopsies in Japan.

Clin Exp Nephrol15: 493-503 , 2011.

19 ) Nakazawa D , Shida H, Tomaru U, Yoshida M, Nishio S, Atsumi T, Ishizu A: Enhanced formation and disordered regulation of NETs in myeloperoxidase-ANCA-associated microscopic polyangiitis. J Am Soc Nephrol25: 990-997,2014.

20 ) O'Sullivan KM, Lo CY, Summers SA, Elgass KD, McMillan PJ, Longano A, Ford SL, Gan PY, Kerr PG, Kitching AR, Holdsworth SR: Renal participation of myeloperoxidase in antineutrophil cytoplasmic antibody (ANCA)-associated glomerulonephritis. Kidney Int88: 1030-1046, 2015.

21 ) Saffarzadeh M, Juenemann C, Queisser MA, Lochnit G, Barreto G, Galuska SP, Lohmeyer J, Preissner KT: Neutrophil extracellular traps directly induce epithelial and endothelial cell death: a predominant role of histones. PLoS One7: e32366,2012.

22 ) Nakazawa D, Kumar SV, Marschner J, Desai J, Holderied A, Rath L, Kraft F, Lei Y, Fukasawa Y, Moeckel GW, Angelotti ML, Liapis H, Anders HJ: Histones and Neutrophil Extracellular Traps Enhance Tubular Necrosis and Remote Organ Injury in Ischemic AKI. J Am Soc Nephrol28: 1753-1768,2017.

23 ) Brinkmann V, Reichard U, Goosmann C, Fauler B, Uhlemann Y, Weiss DS, Weinrauch Y, Zychlinsky A. :Neutrophil extracellular traps kill bacteria. Science303: 1532-1535,2004.

24 ) Rosin DL, Okusa MD: Dangers within: DAMP responses to damage and cell death in kidney disease. J Am Soc Nephrol22: 416-425,2011.

25 ) Takahashi-Iwanaga H: The three-dimensional cytoarchitecture of the interstitial tissue in the rat kidney. Cell Tissue Res264: 269-281,1991.

26 ) Guardiola J, Lobatón T, Rodríguez-Alonso L, Ruiz-Cerulla A, Arajol C, Loayza C, Sanjuan X, Sánchez E, Rodríguez-Moranta F: Fecal level of calprotectin identifies histologic inflammation in patients with ulcerative colitis in clinical and endoscopic remission. Clin Gastroenterol Hepatol12: 1865-1870,2014.

27 ) Yamaguchi S, Takeuchi Y, Arai K, Fukuda K, Kuroki Y, Asonuma K, Takahashi H, Saruta M, Yoshida H: Fecal calprotectin is a clinically relevant biomarker of mucosal healing in patients with quiescent ulcerative colitis. J Gastroenterol Hepatol31: 93-98,2016.

28 ) Theede K, Holck S, Ibsen P, Kallemose T, Nordgaard-Lassen I, Nielsen AM.: Fecal Calprotectin Predicts Relapse and Histological Mucosal Healing in Ulcerative Colitis. Inflamm Bowel Dis22: 1042-1048,2016.

29 ) Pepper RJ, Hamour S, Chavele KM, Todd SK, Rasmussen N, Flint S, Lyons PA, Smith KG, Pusey CD, Cook HT, Salama AD: Leukocyte and serum S100A8/S100A9 expression reflects disease activity in ANCA-associated vasculitis and glomerulonephritis. Kidney Int83: 1150-1158,2013.

30 ) Tyden H, Lood C, Gullstrand B, Jönsen A, Nived O, Sturfelt G, Truedsson L, Ivars F, Leanderson T, Bengtsson AA: Increased serum levels of S100A8/A9 and S100A12 are associated with cardiovascular disease in patients with inactive systemic lupus erythematosus. Rheumatology (Oxford)52: 2048-2055,2013.

31 ) Lood C, Tydén H, Gullstrand B, Jönsen A, Källberg E, Mörgelin M, Kahn R, Gunnarsson I, Leanderson T, Ivars F, Svenungsson E, Bengtsson AA: Platelet-Derived S100A8/A9 and Cardiovascular Disease in Systemic Lupus Erythematosus. Arthritis Rheumatol68: 1970-1980,2016.

32 ) Lichtnekert J, Kulkarni OP, Mulay SR, Rupanagudi KV, Ryu M, Allam R, Vielhauer V, Muruve D, Lindenmeyer MT, Cohen CD, Anders HJ: Anti-GBM glomerulonephritis involves IL-1 but is independent of NLRP3/ASC inflammasome-mediated activation of caspase-1. PLoS One6: e26778,2011.

33 ) Anders HJ, Muruve DA: The inflammasomes in kidney disease. J Am Soc Nephrol22: 1007-1018,2011.

34 ) Misawa T, Takahama M, Kozaki T, Lee H, Zou J, Saitoh T, Akira S.: Microtubule-driven spatial arrangement of mitochondria promotes activation of the NLRP3 inflammasome. Nat Immunol14: 454-460,2013.

35 ) Cronstein BN, Terkeltaub R: The inflammatory process of gout and its treatment. Arthritis Res Ther8 Suppl 1: S3,2006.

36 ) Baur JA, Sinclair DA: Therapeutic potential of resveratrol: the in vivo evidence. Nat Rev Drug Discov5: 493-506,2006.

37 ) Misawa T, Saitoh T, Kozaki T, Park S, Takahama M, Akira S: Resveratrol inhibits the acetylated alpha-tubulin-mediated assembly of the NLRP3-inflammasome. Int Immunol27: 425-434,2015.

38 ) Coll RC, Robertson AA, Chae JJ, Higgins SC, Muñoz-Planillo R, Inserra MC, Vetter I, Dungan LS, Monks BG, Stutz A, Croker DE, Butler MS, Haneklaus M, Sutton CE, Núñez G, Latz E, Kastner DL, Mills KH, Masters SL, Schroder K, Cooper MA, O'Neill LA.: A small-molecule inhibitor of the NLRP3 inflammasome for the treatment of inflammatory diseases. Nat Med21: 248-255,2015.

39 ) van Hout GP, Bosch L, Ellenbroek GH, de Haan JJ, van Solinge WW, Cooper MA, Arslan F, de Jager SC, Robertson AA, Pasterkamp G, Hoefer IE: The selective NLRP3-inflammasome inhibitor MCC950 reduces infarct size and preserves cardiac function in a pig model of myocardial infarction. Eur Heart J38: 828-836,2017.

40 ) Menu P, Vince JE: The NLRP3 inflammasome in health and disease: the good, the bad and the ugly. Clin Exp Immunol166: 1-15,2011.

41 ) Ridker PM, MacFadyen JG, Glynn RJ, Koenig W, Libby P, Everett BM, Lefkowitz M, Thuren T, Cornel JH: Inhibition of Interleukin-1beta by Canakinumab and Cardiovascular Outcomes in Patients With Chronic Kidney Disease. J Am Coll

Cardiol71: 2405-2414,2018.

**Legends for Figures**

**Fig. 1: The correlation between GI and TII in IgAN and ANCAGN.**

For severity quantitation of GI and TII, cCFR and cTII are evaluated from the histological findings of renal biopsy specimen of each case and they are plotted to examine the correlation between them.

**Fig. 2: Light microscopy of the kidney biopsy.**

Immunohistochemical study revealed calprotectin positive cells are seen within active crescents and in areas of focal necrosis. **(A)** ANCAGN, **(B)** IgAN, **(C)** around a small vessel in ANCAGN.

**Fig. 3: The correlation between GCP and TICP scores in IgAN and ANCAGN.**

GCP and TICP scores are evaluated from the immunohistological findings of renal biopsy specimen of each case and they are plotted to examine the correlation between them.

**Fig. 4: The correlation between cTII and TICP scores, and mRNA expression level of *TLR4* and TICP score in ANCAGN.**

**Fig. 5: The correlation of cTII with mRNA expression level of *TLR4, NLRP3, and IL-1β* in ANCAGN.**

A. The correlation between cTII and mRNA expression level of *TLR4*.

B. The correlation between cTII and mRNA expression level of *NLRP3*.

C. The correlation between cTII and mRNA expression level of *IL-1β*.

**Fig. 6: Immnofluoresecnt study by Mantra Quantitative Pathology Imaging System shows the distribution of infiltrating cells.**

Calprotectin deposits (**cyan**), NLRP3-positive cells (**magenta**), TLR4-positive cells (**green**), and nucleus (**blue**) are shown in the tubulointerstitium.

**The authors declare that no conflict of interest.**

Table 1. Patients characteristics at the time of kidney biopsy

| | ANCA(n=22) | IgAN(n=25) |
|---|---|---|
| Age | 65.6±15.1 | 38.5±14.6 |
| Sex | Male: 12 Female: 10 | Male: 10 Female: 15 |
| WBC (/μL) | 12,531 ± 19,290 | 7,246 ± 3,194 |
| TP (g/dl) | 6.66 ± 0.81 | 5.81 ± 2.22 |
| Alb (g/dl) | 2.59 ± 0.80 | 4.85 ± 11.3 |
| BUN (mg/dl) | 34.4 ± 19.5 | 14.8 ± 7.41 |
| Cr (mg/dl) | 9.98 ± 4.12 | 0.91 ± 0.57 |
| eGFR (ml/min/1.73m$^2$) | 28.78 ± 26.5 | 57.32 ± 31.68 |
| CRP (mg/dl) | 8.00 ± 6.71 | 0.21 ± 0.45 |
| MPO-ANCA (U/ml) | 189.3 ± 187.6 | not examined |
| IgG (mg/dl) | 1,547 ± 601 | 916 ± 475 |
| U-Protein (g/day) | 1.34 ± 1.30 | 1.67 ± 1.65 |
| U-β2MG (μg/l) | 14,414 ± 18,404 | 1,250 ± 3,234 |
| U-NAG (IU/l) | 19.3±13.0 | 14.6 ± 18.3 |

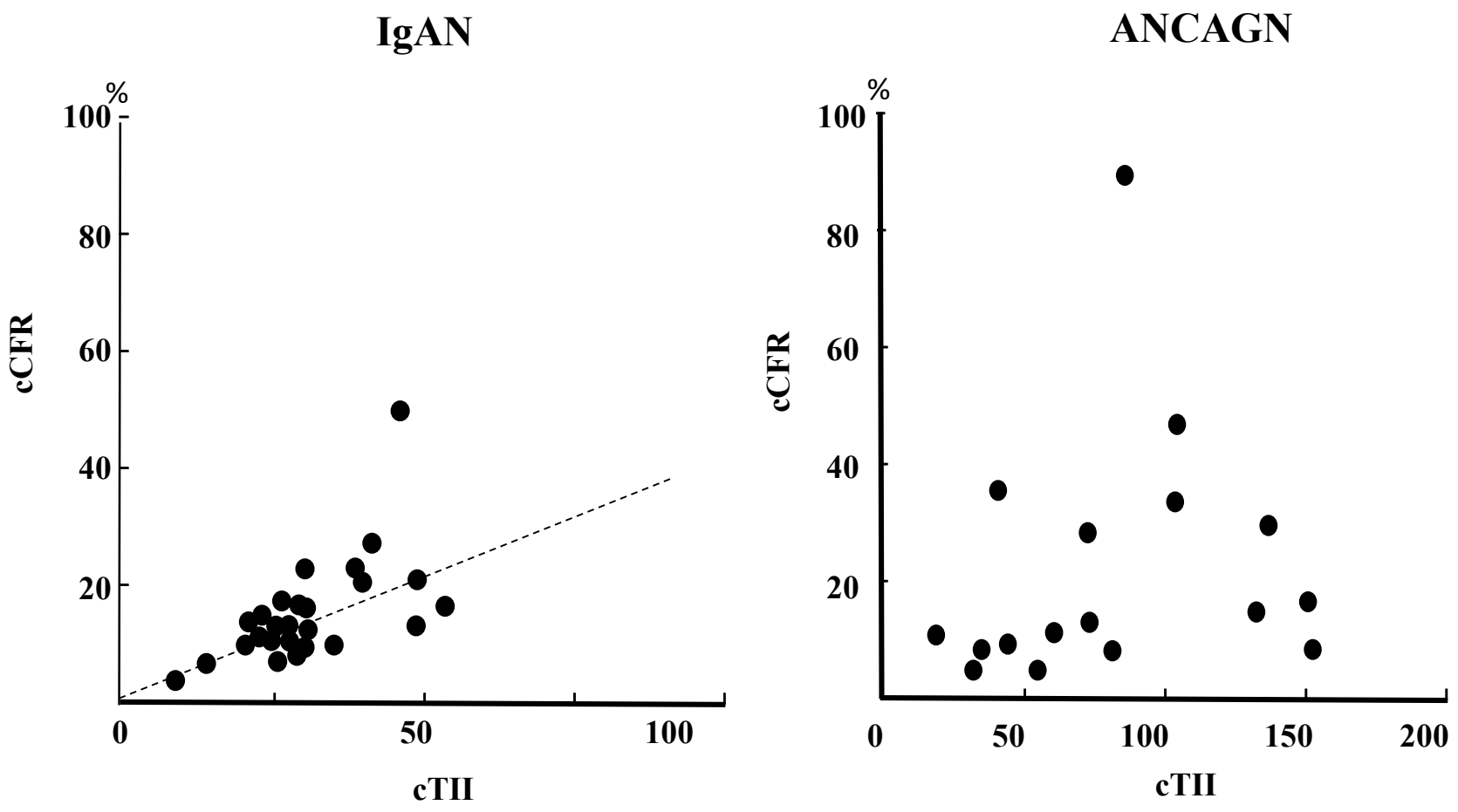
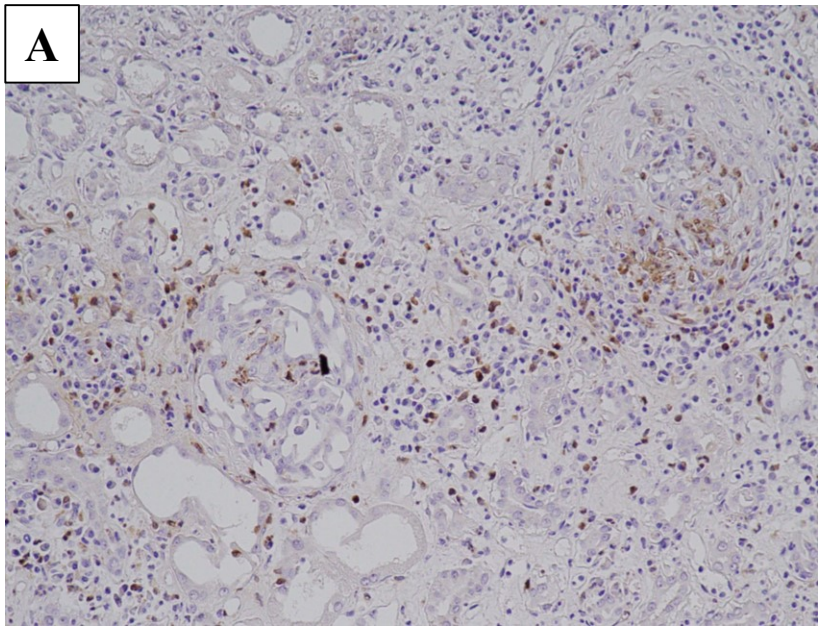
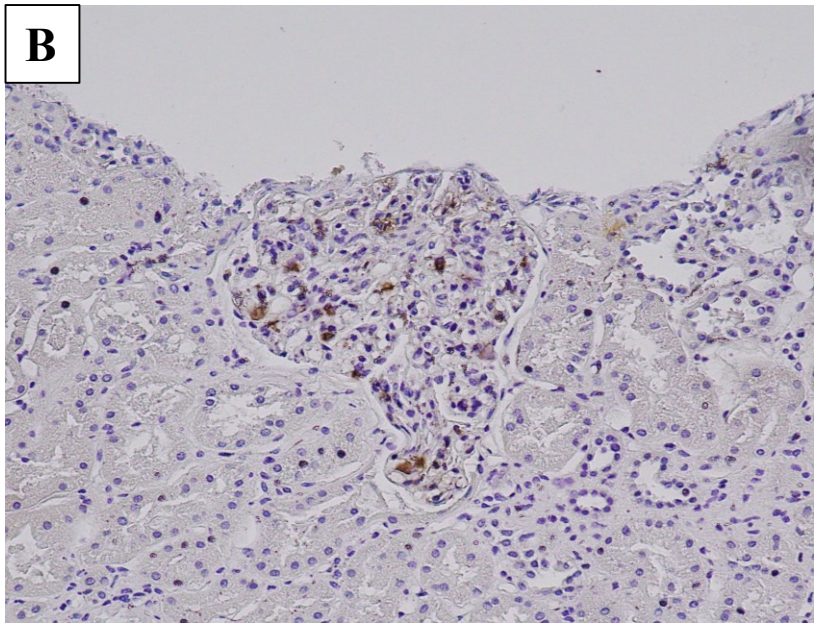

IgAN
%
100
80
60
40
20
cCFR
0
50
100
cTII
ANCAGN
%
100
80
60
40
20
cCFR
0
50
100
150
200
cTII



**Figure 1**



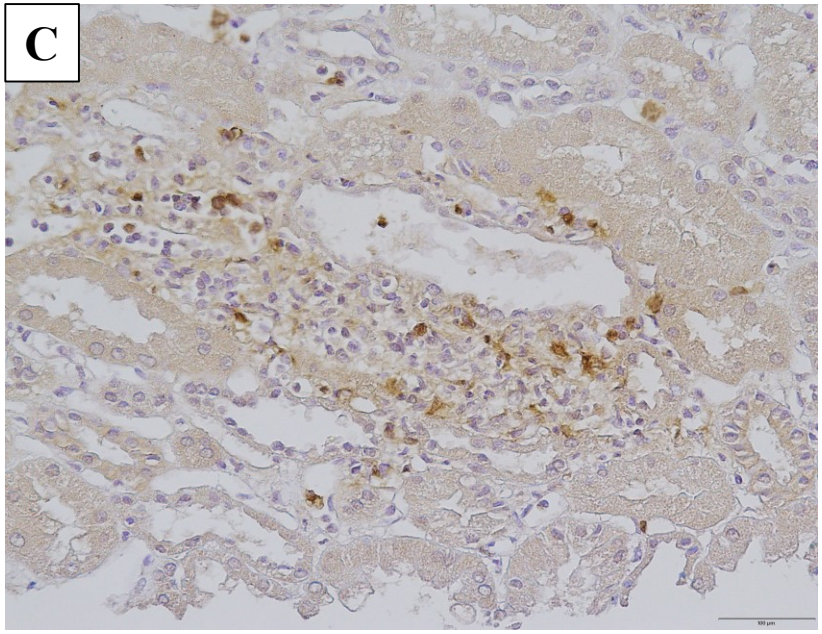
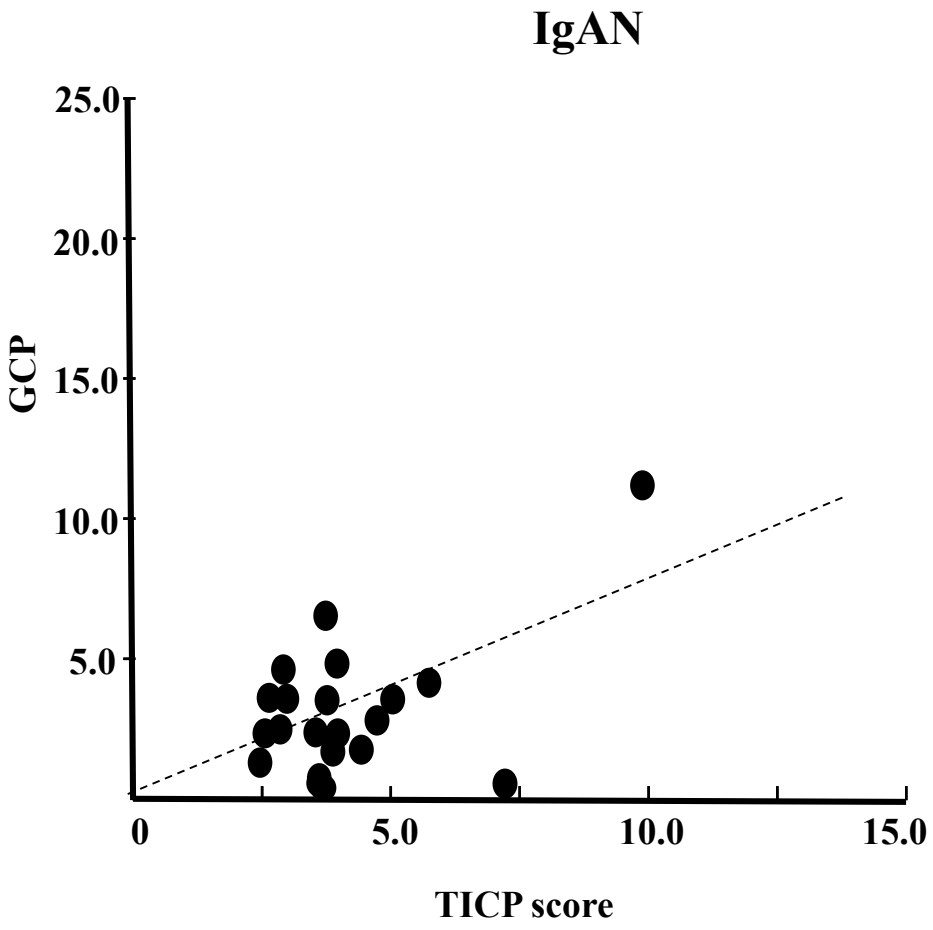






**Figure 2**


IgAN
25.0
20.0
15.0
10.0
5.0
GCP
0
5.0
10.0
15.0
TICP score

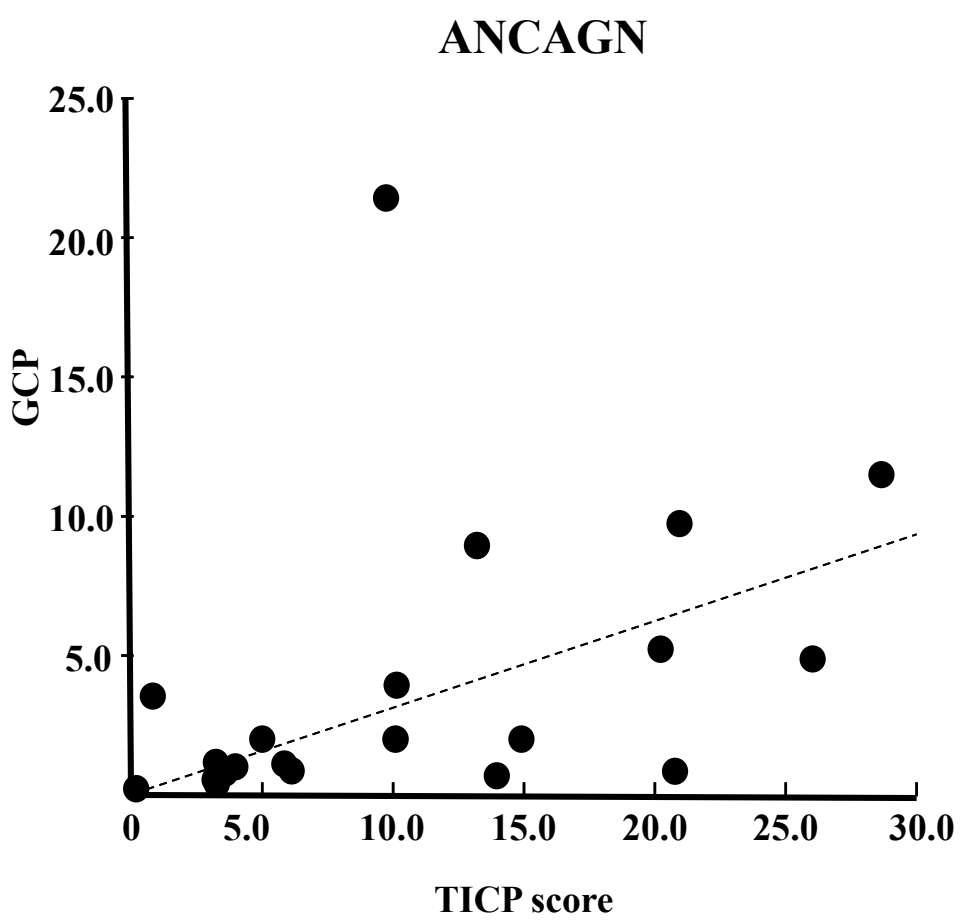

ANCAGN
25.0
20.0
15.0
10.0
5.0
GCP
0
5.0
10.0
15.0
20.0
25.0
30.0
TICP score



**Figure 3**

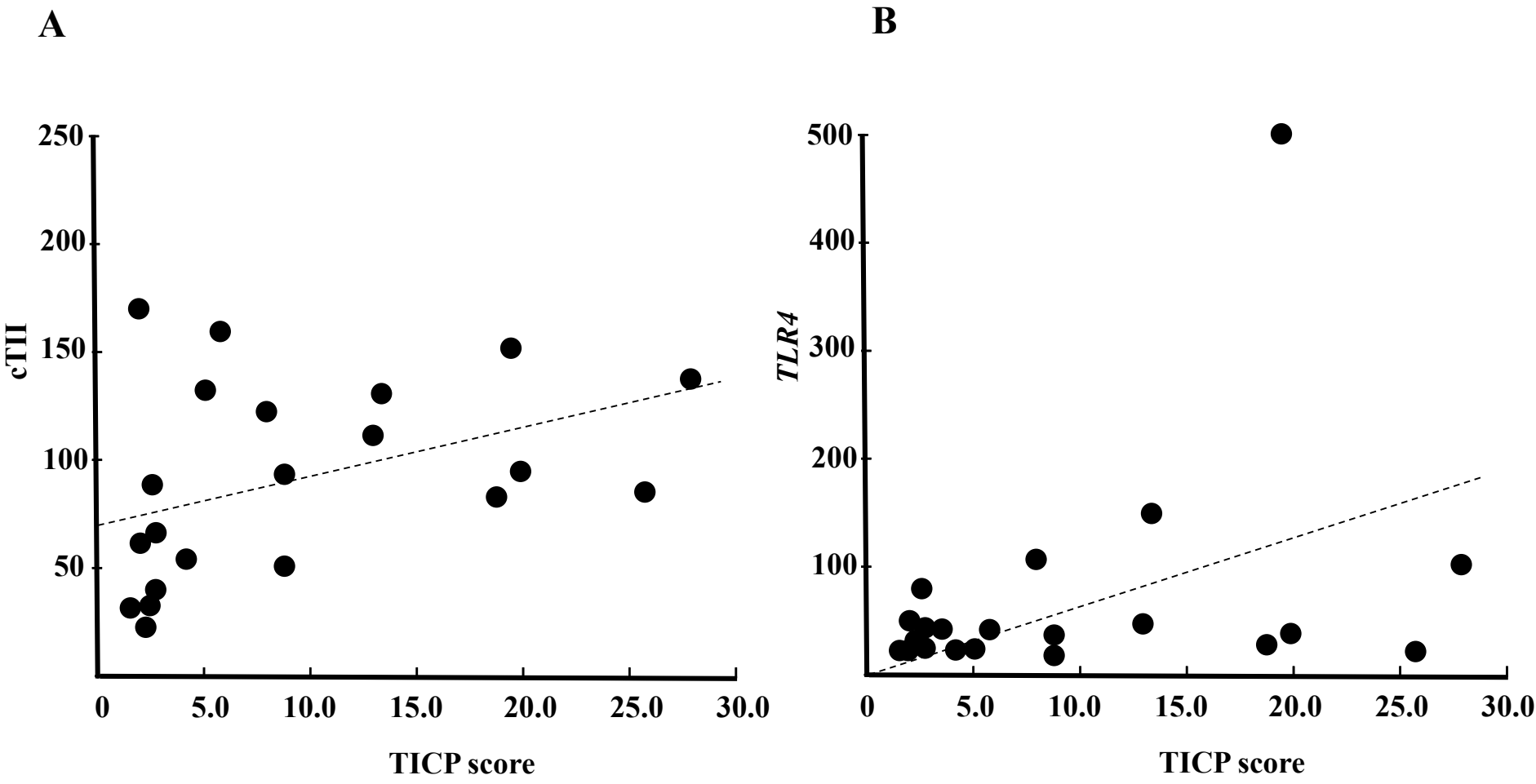

A
250
200
150
100
50
cTII
0
5.0
10.0
15.0
20.0
25.0
30.0
TICP score
B
500
400
300
200
100
TLR4
0
5.0
10.0
15.0
20.0
25.0
30.0
TICP score



**Figure 4**

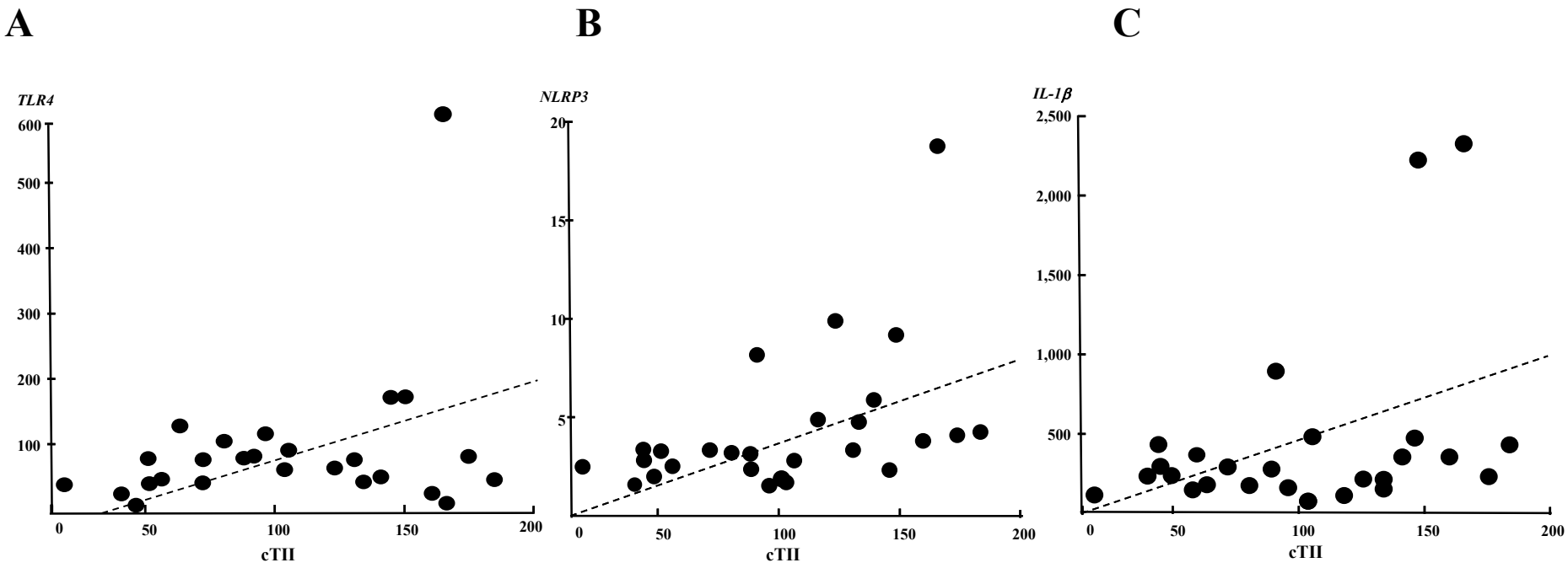

A
TLR4
600
500
400
300
200
100
0
50
100
150
200
cTII
B
NLRP3
20
15
10
5
0
50
100
150
200
cTII
C
IL-1β
2,500
2,000
1,500
1,000
500
0
50
100
150
200
cTII



**Figure 5**

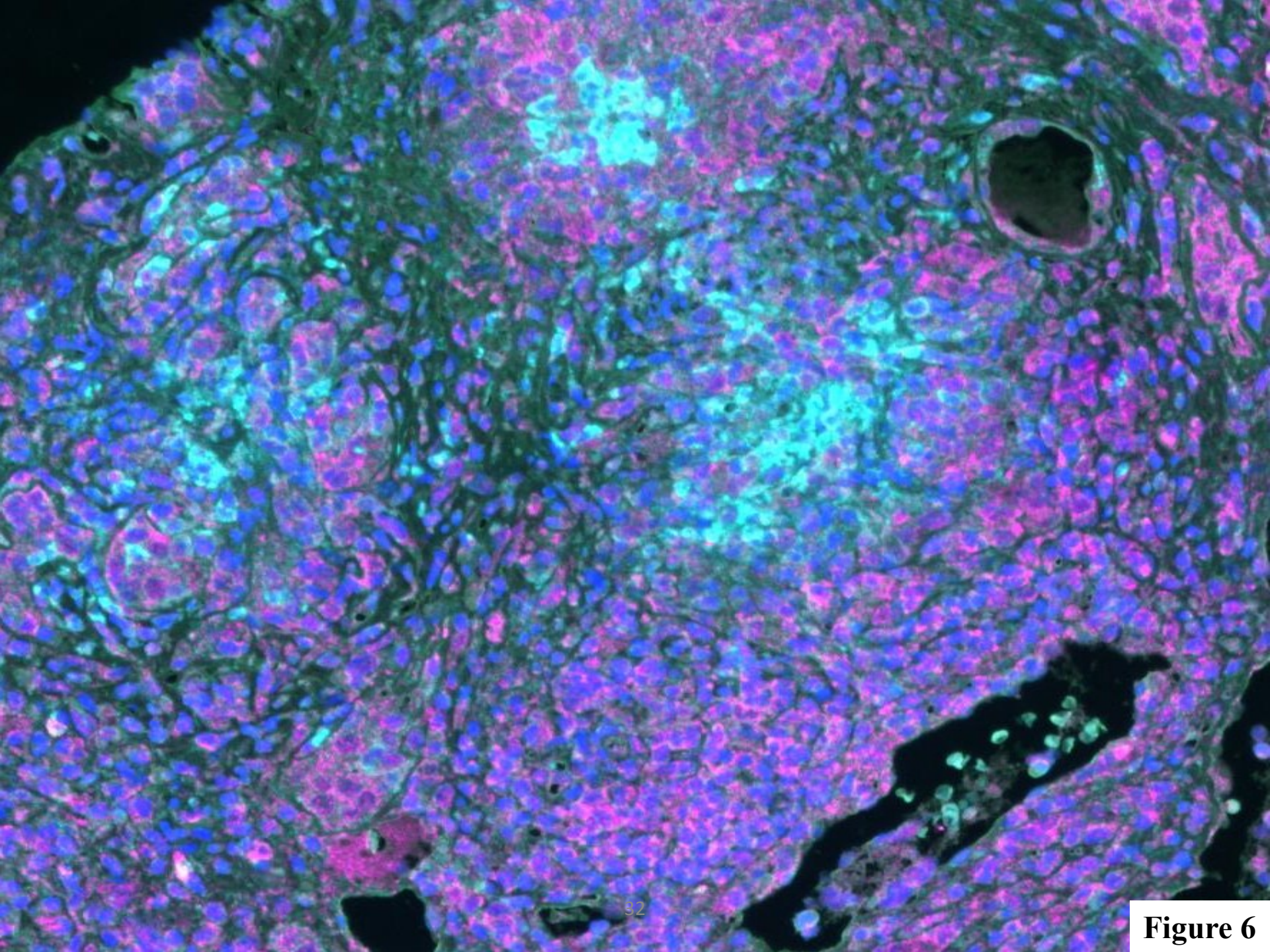




**Figure 6**